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Short communication

Cross-protective *Salmonella* vaccine reduces cecal and splenic colonization of multidrug-resistant *Salmonella enterica* serovar Heidelberg

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A B S T R A C T

Salmonella vaccine strategies for food-producing animals have typically focused on a specific serovar that either causes production losses due to morbidity/mortality or is an important food safety pathogen for a particular food commodity. The *Salmonella enterica* serovar Typhimurium BBS 866 vaccine strain was designed to reduce serovar specificity to provide cross-protection against diverse *Salmonella* serovars, thereby broadening its applicability for multiple animal and poultry species. We reported cross-protection of the BBS 866 vaccine in swine [*Vaccine 34:1241–6*]. In the current study, we extend the efficacy of the *Salmonella* vaccine to a poultry commodity by revealing significant reduction of multidrug-resistant *Salmonella enterica* serovar Heidelberg colonization of the cecum and systemic dissemination to the spleen in BBS 866-vaccinated turkeys. Transcriptional analysis of whole blood from BBS 866-vaccinated turkeys revealed down-regulation of metabolic and immune genes (*KCNAB1, ACOD1, GPR17, ADOR2AB,* and *IL-17RD*), suggesting limited leukocyte activation without an overt peripheral inflammatory response to vaccination.

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1. Introduction

Salmonella is a leading cause of human foodborne disease, and an estimated 17% of human infections with *Salmonella* in the U.S. are attributed to the consumption of contaminated turkey [1]. In 2011, a foodborne illness outbreak due to multidrug-resistant (MDR; resistant to $\geq$3 antimicrobial classes) *Salmonella* from ground turkey sickened 136 individuals (1 death) and resulted in the recall of 36 million pounds of ground turkey, one of the largest meat recalls in U.S. history [2]. Pre-harvest interventions are needed to reduce the entrance of *Salmonella* into the human food chain, but a challenge in controlling *Salmonella* in the food supply is undetected colonization of food-producing animals. Thus, a critical control point for strategic intervention is *Salmonella* reduction in asymptomatic carrier animals.

Vaccination is a viable option for reduction of human foodborne pathogens in food animals to prevent foodborne outbreaks and limit product losses due to recalls. Recently, we designed a live, attenuated *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) oral vaccine (BBS 866; Δ*rybB* Δ*omrAB* Δ*micA* Δ*invR rfaH::neo*) for use in food-producing animals [3]. The combination of mutations in the BBS 866 vaccine reduces serovar-specific lipopolysaccharide (LPS) while enhancing the exposure of conserved outer membrane proteins (OMPs) of *Salmonella*; thus, reduction of LPS in BBS 866 limits the serovar-specific immune response following vaccination while OMPs provide immune targets conserved across diverse *Salmonella* serovars. Our evaluation of BBS 866 in swine demonstrated reduced disease and colonization due to either serovar Choleraesuis or Typhimurium in vaccinated compared to mock-vaccinated pigs [3,4]. Because these investigations in swine established that the BBS 866 vaccine provides not only homologous protection against *S.* Typhimurium but also cross-protection against a heterologous serovar (Choleraesuis), the use of the vaccine may be more broadly applicable to multiple animal and poultry species.

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In the current study, we evaluated the *Salmonella* BBS 866 vaccine in turkeys for reduction of a MDR *S.* Heidelberg isolate from the 2011 ground turkey outbreak. Vaccinated turkeys that were subsequently challenged with *S.* Heidelberg had significantly reduced *S.* Heidelberg colonization in the cecum and systemic dissemination to the spleen compared to non-vaccinated turkeys. Transcriptional analysis of peripheral blood leukocytes from 3-week old turkeys at 2 days post-vaccination indicated limited pro-inflammatory signaling and leukocyte activation. Collectively, the data demonstrate an efficacy of the *Salmonella* vaccine for reduction of MDR *S.* Heidelberg colonization in turkeys.

2. Materials and methods

2.1. Bacterial strains

BBS 866 is a live, attenuated *S. enterica* serovar Typhimurium vaccine strain that has been previously described [3]. Multidrug-resistant *S.* Heidelberg strain BSX 126 (2011K-1138; CVM41579) isolated from ground turkey associated with a 2011 outbreak is resistant to ampicillin, tetracycline, streptomycin, and gentamicin [5]. BSX 126 was inoculated into a turkey, isolated from the cecum at 7 days post-inoculation (dpi), and designated SB 392. SB 392 is a weak H_2S producer; thus, the tergitol concentration in XLT-4 (Beckton, Dickinson and Co., Sparks, MD, USA) was reduced to 25% of the normal level, thereby allowing the isolate to produce H_2S [6]. Bacterial growth medium for isolation of SB 392 from turkeys was XLT-4 containing 25% tergitol (1.15 ml/L), tetracycline (15 μg/ml), streptomycin (50 μg/ml), and novobiocin (40 μg/ml). Isolation of BBS 866 from turkeys was performed using XLT4 containing kanamycin (50 μg/ml) and nalidixic acid (30 μg/ml).

2.2. Animal trials and sampling procedures

Experiment 1. One-day old male turkey poults (n = 16) were group housed for two weeks. Pen fecal samples tested negative for *Salmonella* twice using qualitative bacteriology as previously described [4]. Turkeys were separated into individual pens and inoculated by oral gavage with 10^8 (n = 8) or 10^{10} (n = 8) CFU (colony forming units) of the *Salmonella* BBS 866 vaccine at 3-weeks of age. At 0, 1, 2, and 3 days post-vaccination (dpv) cloacal temperatures were measured using a Medline thermometer, model # MDS9850B (Mundelein, IL). At 0, 1, 2, 3, 7, 10, and 14 dpv, *Salmonella* levels in the feces were determined using quantitative and qualitative bacteriology as previously described [4]. Four turkeys from the 10^8 and the 10^{10} vaccinated groups at 7 and 14 dpv were euthanized and tissues [crop, liver, spleen, cecum, bursa of Fabricius and cloaca] were collected for *Salmonella* BBS 866 enumeration as previously described [4].

Experiment 2. Two groups (n = 12/group) of one-day old male turkey poults were housed in separate isolation rooms at the National Animal Disease Center. At 2-days of age, turkey poults received an oral gavage of 500 μl of PBS (mock-vaccinated) or PBS containing 4×10^8 CFU of the *Salmonella* BBS 866 vaccine. A total of three poults died between vaccination and booster vaccination (unrelated to vaccination). At 2-weeks of age, respective poults received a 1 ml booster of either PBS (n = 10) or PBS containing 1×10^9 CFU of BBS 866 (n = 11). At 5-weeks of age, all turkeys were challenged via oral gavage with 1×10^9 CFU of MDR *S.* Heidelberg SB 392. Seven days after challenge, all turkeys were euthanized, and their spleen and ceca were harvested for *Salmonella* enumeration.

Procedures involving animals followed humane protocols as approved by the USDA, ARS, National Animal Disease Center Animal Care and Use Committee in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Research Council of the National Academies.

2.3. RNA isolation from blood and RNASeq analysis

Prior to vaccination and 2 days post-vaccination with 10^{10} CFU of BBS 866 (experiment 1), 9 ml of blood was collected from the wing vein of individual 3-week-old turkey toms (n = 3/time point) using the LeukoLOCK Fractionation & Stabilization Kit (ThermoFisher Scientific), total RNA from the leukocyte population was isolated using the LeukoLOCK Total RNA Isolation System (ThermoFisher Scientific), and RNASeq analysis performed as previously described [6]. Briefly, libraries were constructed using the Illumina TruSeq RNA Sample Prep Kit v2 and sequenced on an Illumina HiSeq 2500 in a 100-cycle paired-end sequencing run. Using CLC Genomic workbench V 9.5.2, sequences were trimmed, mapped to the 23,976 predicted genes in the Meleagris gallopavo reference assembly 5.0 [7], and empirical analysis of differential gene expression was performed using the EdgeR statistical test on the raw unique reads [8]. Gene expression differences with greater than 1.5-fold change and a False Discovery Rate (FDR) P-value less than 0.05 were considered significant.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA). One-way analysis of variance with a Dunnett's Multiple Comparison Test was used to analyze body temperatures at 1, 2, and 3 dpv compared to day 0 in turkeys inoculated with BBS 866. Statistical analysis of *S.* Heidelberg quantitation in tissues was performed using an unpaired *t*-test. Contingency analysis of the prevalence of *S.* Heidelberg in the spleen was performed using the Fisher's exact test.

3. Results and discussion

3.1. Fecal shedding and tissue colonization of the Salmonella BBS 866 vaccine strain in turkeys

To evaluate the colonization potential of the *Salmonella* BBS 866 vaccine strain in turkeys, three-week old poults were inoculated

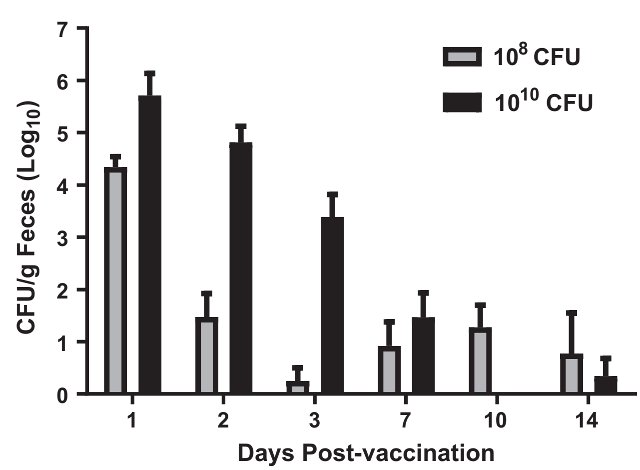

Fig. 1. Fecal shedding of *Salmonella enterica* serovar Typhimurium BBS 866 vaccine strain from turkeys. At 3 weeks of age, individually housed turkeys were inoculated with 10^8 ($n = 8$) or 10^{10} ($n = 8$) CFU of BBS 866. Quantitative and qualitative bacteriology was performed on feces collected at the indicated time points to determine fecal shedding of the vaccine strain. At day 7 post-vaccination, 4 turkeys from each group were euthanized resulting in smaller groups for the remaining time points. Error bars indicate SEM.

with 10^8 or 10^{10} CFU. Fecal shedding of *Salmonella*, body (cloacal) temperature, and tissue colonization were monitored. At either vaccine dose, no increase in average body temperature was observed at 1, 2, or 3 dpv compared to their pre-vaccination body temperature, indicating that the *Salmonella* vaccine strain does not induce a fever in turkeys (average body temperature range at 0, 1, 2, 3 dpv was 41.38–41.60 °C and 41.26–41.56 °C for poults inoculated with 10^8 and 10^{10} CFU, respectively). Fecal shedding of the vaccine strain was detected throughout the 14-day study at both inoculation doses (Fig. 1). The 10^{10} CFU inoculated turkeys shed 1–3 logs more *Salmonella* than the 10^8 inoculated turkeys during the first three days after vaccination, but similar fecal shedding levels were observed between the two treatment groups at 7 and 14 dpv.

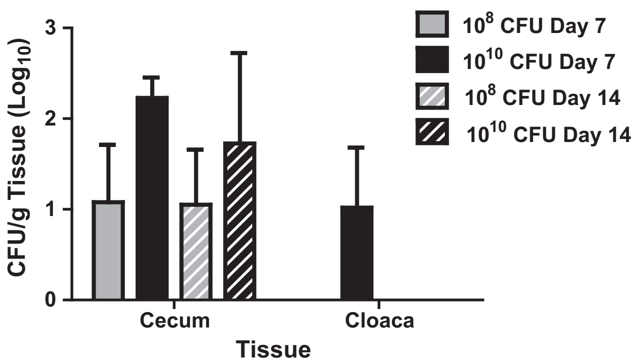

Fig. 2. Tissue colonization of *Salmonella enterica* serovar Typhimurium BBS 866 vaccine strain in turkeys. At 3 weeks of age, individually housed turkeys were inoculated with 10^8 ($n = 8$) or 10^{10} ($n = 8$) CFU of BBS 866. Four turkeys from each group were euthanized at 7 and 14 days following vaccination. The cecum and cloaca were harvested following euthanasia for quantitative and qualitative bacteriology to determine tissue colonization by BBS 866. Error bars indicate SEM.

At 7 and 14 days post-vaccination, tissue colonization of the BBS 866 vaccine was evaluated by quantitative and qualitative (enrichment) bacteriology (Fig. 2). At 7 dpv, the vaccine strain was detected in the cecum (10^8 and 10^{10} doses; 2/4 and 4/4 birds, respectively) and cloaca (10^{10} dose, 2/4 birds). At 14 dpv, the vaccine strain was only observed in the cecum (10^8 and 10^{10} doses; 2/4 and 2/4 birds). The vaccine strain was not detected in the crop, liver, spleen, or bursa of Fabricius at either time point regardless of inoculation dose.

3.2. Transcriptional response of vaccinated turkeys

Prior to vaccination and at 2 days post-vaccination, peripheral blood leukocytes were obtained from three turkeys to evaluate the gene expression response to the BBS 866 vaccine (day 2/day 0). Twenty-six differentially expressed genes were significantly down-regulated ($P < 0.05$) in response to vaccination, including predicted immune regulatory genes *KCNAB1, ACOD1, GPR17* and *IL-17RD* (Table 1). IL-17RD negatively regulates IL-17A-induced activation of NF-κB and decreases the expression of pro-inflammatory cytokine genes, including IL-6 [9]. ACOD1, also known as immunoresponsive gene 1 or *cis*-aconitate decarboxylase, up regulates expression of A20, which prevents the production of TLR-induced pro-inflammatory cytokines [10]. KCNAB1 is a potassium channel, voltage-gated ion channel and ADOR2AB is a receptor for adenosine, both of which may be involved in lymphocyte activation as well as naïve and regulatory T cell development [11–13]. Overall, vaccine efficacy to reduce *S.* Heidelberg colonization (see data below) was achieved without producing a strong pro-inflammatory immune response in peripheral blood leukocytes. This limited immune response may be attributed to BBS 866 being a DIVA vaccine (Differentiation of Infected from Vaccinated Animals) due to a mutation in the *rfaH* gene that substantially reduces lipopolysaccharide (LPS) production [3,14]. As a

Table 1
Meleagris gallopavo (turkey) genes differentially expressed in response to vaccination with *Salmonella enterica* serovar Typhimurium strain BBS 866 (2 dpv/0 dpv).

Gene Symbol	Fold change	FDR P-value	Ensembl[a]	Source	Predicted Gene Description
KCNAB1	−13.73	1.11E−05	10715	HGNC Symbol; Acc:HGNC:6228	Potassium voltage-gated channel subfamily A member regulatory beta subunit 1
SCG3	−7.91	3.52E−14	06516	HGNC Symbol; Acc:HGNC:13707	Secretogranin III
ADAMTS6	−6.71	0.0076	05112	HGNC Symbol; Acc:HGNC:25835	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 6
BPGM	−3.89	0.0362	13525	HGNC Symbol; Acc:HGNC:1093	Bisphosphoglycerate Mutase
HESX1	−3.54	0.0076	06238	HGNC Symbol; Acc:HGNC:4877	HESX Homeobox 1
SLC41A3	−3.42	0.0063	07576	HGNC Symbol; Acc:HGNC:31046	Solute Carrier Family 41 Member 3
IL17RD	−3.38	0.0372	06214	HGNC Symbol; Acc:HGNC:17616	Interleukin 17 Receptor D
FGFR3	−3.1	0.0076	13059	HGNC Symbol; Acc:HGNC:3690	Fibroblast growth factor receptor 3
DDX11	−2.94	0.0076	13363	HGNC Symbol; Acc:HGNC:2736	DEAD/H-Box Helicase 11
ACOD1	−2.67	0.0146	14936	HGNC Symbol; Acc:HGNC:33904	Aconitate Decarboxylase 1
ZSWIM7	−2.49	0.0288	06262	HGNC Symbol; Acc:HGNC:26993	Zinc Finger SWIM-Type Containing 7
ADORA2B	−2.47	0.0008	06254	HGNC Symbol; Acc:HGNC:264	Adenosine A2b Receptor
GPR17	−2.27	0.0431	15621	HGNC Symbol; Acc:HGNC:4471	G Protein-Coupled Receptor 17
LOC100547913	−2.09	0.0063	01744	HGNC Symbol; Acc:HGNC:636	Aquaporin-3 (AQP3)
ABAT	−2.06	0.0076	08563	HGNC Symbol; Acc:HGNC:23	4-aminobutyrate aminotransferase
LOC100548279	−2.05	0.0063	14251	NCBI gene; Acc:100548279	Cytochrome P450 2K4-like
SLC16A10	−2.03	0.0033	13427	HGNC Symbol; Acc:HGNC:17027	Solute Carrier Family 16 Member 10
ASBD9	−2.02	0.0455	14696	HGNC Symbol; Acc:HGNC:17184	Ankyrin repeat and SOCS box protein 9
STK35	−1.99	0.0431	06965	HGNC Symbol; Acc:HGNC:16254	Serine/Threonine Kinase 35
FAM207A	−1.92	0.0314	01046	HGNC Symbol; Acc:HGNC:15811	Family With Sequence Similarity 207 Member A
ODC1	−1.87	0.0288	14011	HGNC Symbol; Acc:HGNC:8109	Ornithine decarboxylase 1
STARD4	−1.86	0.0288	08766	HGNC Symbol; Acc:HGNC:18058	StAR Related Lipid Transfer Domain Containing 4
LOC100551072	−1.79	0.0348	08803	NCBI gene; Acc:100551072	Hemoglobin subunit alpha-D
TXN2	−1.76	0.0122	12649	HGNC Symbol; Acc:HGNC:17772	Thioredoxin 2
LOC100545668	−1.71	0.0288	13513	RefSeq: XM_003202470	Aldose reductase-like
GCHFR	−1.64	0.0431	02945	HGNC Symbol; Acc:HGNC:4194	GTP Cyclohydrolase I Feedback Regulator

[a] The Ensembl number is proceeded by ENSMGAG000000.

result, the BBS 866 vaccine may not activate the Toll-like receptor that recognizes LPS (in fact, *TLR3, TLR4, TLR5* and *TLR7* were not differentially expressed), thereby potentially explaining the lack of differential expression of pro-inflammatory genes (e.g. *IFNG, IL-6, IL-8*, etc.). Of the 26 differentially expressed genes in response to vaccination, six of the genes were similarly down-regulated in age-matched turkeys challenged with 10^{10} CFU of MDR *S.* Heidelberg (*KCNAB1, SCG3, ODC1, FAM207A, LOC100547913,* and *LOC100545668*) [6]. Further studies are needed to investigate the mucosal immune response in intestinal tissues after vaccination. Although performance measurements were not collected in these turkeys, the lack of an overt peripheral inflammatory response and fever after vaccination suggests that a production impact from vaccination would not be anticipated.

3.3. Vaccination reduces MDR S. Heidelberg in the cecum and spleen of challenged turkeys

In a second trial, turkey poults were vaccinated (2 days old) and booster vaccinated (2 weeks old) with the *S.* Typhimurium BBS 866 vaccine (n = 11); age-matched turkeys were mock-vaccinated with PBS (n = 10). Both turkey groups were challenged with 1×10^9 CFU of MDR *S.* Heidelberg at 5 weeks of age. At 7 days post-challenge, *S.* Heidelberg CFU/g of ceca tissue was significantly lower (P = 0.0149) in vaccinated turkeys compared to mock-vaccinated turkeys (Fig. 3). MDR *S.* Heidelberg colonization of the spleen was also evaluated because *Salmonella* isolation from spleen tissue is indicative of a systemic infection. The number of turkeys positive for *S.* Heidelberg in the spleen was significantly lower (P = 0.0019) in vaccinated turkeys compared to mock-vaccinated turkeys (Fig. 3). Furthermore, *S.* Heidelberg CFU/g of spleen tissue was significantly decreased in vaccinated turkeys compared to mock-vaccinated turkeys (P = 0.0002). Although serovars Heidelberg and Typhimurium both belong to Group O:4 (B) based on their antigenic formula, a population structure investigation of 114 diverse *Salmonella enterica* isolates indicated that Heidelberg is in a distinct lineage from Typhimurium [15]. Thus, these results demonstrate that the *S. enterica* serovar Typhimurium BBS 866 vaccine induced an immune response that provided cross-

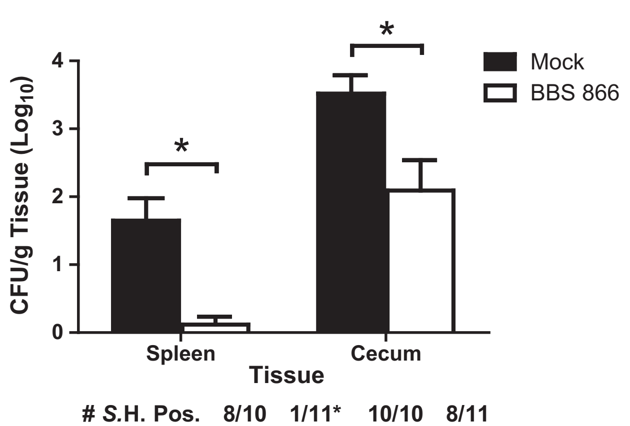

Fig. 3. Reduction of MDR *Salmonella enterica* serovar Heidelberg colonization of the spleen and cecum in BBS 866-vaccinated turkeys. At 2 days and 2 weeks of age, turkey poults were vaccinated with 4×10^8 CFU and 1×10^9 CFU of *Salmonella* BBS 866, respectively. At 5 weeks of age, mock-vaccinated (n = 10) and BBS 866-vaccinated (n = 11) turkeys were challenged with 1×10^9 CFU of MDR *S. enterica* serovar Heidelberg strain SB 392. The cecum and spleen were harvested following euthanasia for quantitative and qualitative bacteriology to determine tissue colonization by SB 392. Error bars indicate SEM. The number of turkeys positive for *Salmonella* Heidelberg (numerator) and the total number of turkeys per group (denominator) is indicated. *Statistically significant differences between groups ($P \leq 0.05$).

protection in turkeys from systemic infection (spleen) and reduced gastrointestinal colonization (cecum) by *S. enterica* serovar Heidelberg.

In summary, intervention strategies in the animal reservoir are necessary to fully optimize comprehensive control programs against *Salmonella* to support food safety, especially as livestock and poultry production systems continue to increase in size and complexity, and antibiotic usage in animal production becomes increasingly controversial. Vaccination against *Salmonella* is an on-farm intervention strategy that can reduce dissemination of the human foodborne pathogen at multiple points along the food chain, including transmission of *Salmonella* from infected animals to naïve animals during food animal production, environmental contamination of crops and waterways when animal manure is used for soil amendment (fertilizer), and contamination of slaughter plants and meat products that can result in food recalls. Our current and published research on the *Salmonella* BBS 866 vaccine demonstrates its effectiveness as a pre-harvest intervention because it provides cross protection against different *Salmonella* serovars in diverse animal species for both gastrointestinal colonization and systemic disease.

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Conflict of interest statement

An international patent has been filed by the U.S. Department of Agriculture (EP14877328.6) and a U.S. patent has been issued (US 9,868,769 B2) for *S.* Typhimurium BBS 866 with BLB and SMDB indicated as co-inventors.

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendations or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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